Annals of Botany **110**: 1291–1301, 2012
doi:10.1093/aob/mcs168, available online at www.aob.oxfordjournals.org

ANNALS OF
BOTANY
Founded 1887

PART OF A HIGHLIGHT ON BREEDING STRATEGIES
FOR FORAGE AND GRASS IMPROVEMENT

Expression and functional characterization of a white clover isoflavone synthase in tobacco

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Received: 10 March 2012 Returned for revision: 11 May 2012 Accepted: 28 May 2012 Published electronically: 22 August 2012

- *Background and Aims Trifolium repens* (white clover) is a valuable component of pastures due to its ability to fix nitrogen. Productivity of *T. repens* is sometimes threatened by insect pests, and it has been suggested that phenylpropanoid-derived isoflavonoids such as formononetin can protect white clover from insect damage. The aim of this study was to isolate and functionally characterize an isoflavone synthase (IFS2_12) from *T. repens* by expressing it in *Nicotiana tabacum* (tobacco), a plant which does not naturally produce isoflavonoids.
- *Methods* To induce anthocyanin production and increase isoflavonoid precursors in tobacco, the tomato R2R3 MYB transcription factor *ANT1* was expressed in tobacco (Nt-ANT1 plants). *IFS2_12* was heterologously expressed in tobacco both transiently and stably, and isoflavonoids in leaf extracts were analysed by liquid chromatography (LC) coupled to mass spectrometry (MS^n). As a positive control, a double construct of soybean *IFS* and alfalfa chalcone isomerase (*IFS/CHI*), which had been previously shown to induce isoflavonoid production in tobacco, was also expressed. Stable transformants expressing *IFS2_12*, soybean/alfalfa *IFS/CHI* and *ANT1* were crossed and the resulting plants were analysed for isoflavonoid production.
- *Key results* Leaves of tobacco plants expressing *ANT1* had a range of phenotypes from mainly green to uniformly bronze coloured. Both transient and stable expression of the *IFS2_12* or *IFS/CHI* constructs resulted in the production of the isoflavonoid genistein and its conjugates. The highest levels (up to 19·2 mg g^{-1} d. wt) accumulated in a progeny of a cross between a purple *ANT1* and a *IFS/ CHI* transformant, while the second highest concentration was found in a plant derived from a selfed *IFS2-12* transformant.
- *Conclusions* It is concluded that the gene *IFS2_12* isolated from *T. repens* encodes an isoflavone synthase. This study paves the way for engineering white clover plants with higher levels of isoflavonoids than naturally found in this species for sufficient insect protection.

Key words: *Trifolium repens*, *Nicotiana tabacum*, isoflavone synthase, isoflavonoid biosynthesis, transient gene expression, liquid chromatography–mass spectrometry, genistein.

INTRODUCTION

Trifolium repens (white clover) is a valuable component of temperate pastures, providing nitrogen fixation to fertilize the soil and high quality nutrition to grazing livestock (Caradus *et al.*, 1996). However, white clover productivity can be reduced by diseases and pests such as *Sitona lepidus* (clover root weevil; CRW), and, to maintain productivity, resistant or tolerant germplasm needs to be developed. Phenylpropanoids play important roles in plant defence (Dixon and Steele, 1999; Yu *et al.*, 2000; Winkel-Shirley, 2001), and isoflavonoids, a class of phenylpropanoids which occur predominantly in legumes, have been shown to contribute to insect resistance (Lane *et al.*, 1987; Simmonds, 2003). Feeding studies with *T. pratense* (red clover) suggest that the isoflavonoids formononetin and biochanin A deter both CRW larvae feeding on roots, and adults feeding on leaves (Gerard *et al.*, 2005; Murray *et al.*, 2007). Compared with red clover, white clover contains much lower levels of formononetin and biochanin A which are insufficient to deter CRW feeding.

Trifolium repens germplasm shows little variation in isoflavonoid content, and attempts to increase isoflavonoids by conventional breeding strategies have not been successful so far. Traditional phenotype-based breeding is limited to the use of available germplasm, and it takes many years to produce a new white clover cultivar with a different phenotype. Marker-assisted selection can speed up this process but is still limited to the available germplasm. Metabolic engineering allows the incorporation and controlled expression of structural or regulatory genes to produce a new biochemical phenotype that may not exist within any of the current genetic diversity of the species of interest, and can thus potentially achieve greater enhancements than conventional breeding (Dixon and Steele, 1999). Isoflavonoids, flavonols, anthocyanins and condensed tannins are derived from phenylpropanoids and share common precursors (Winkel-Shirley, 2001; Fig. 1). The first committed step to flavonoid biosynthesis is catalysed by chalcone synthase (CHS) which condenses three malonyl-CoA and *p*-coumaroyl-CoA to tetrahydroxychalcone (Austin and

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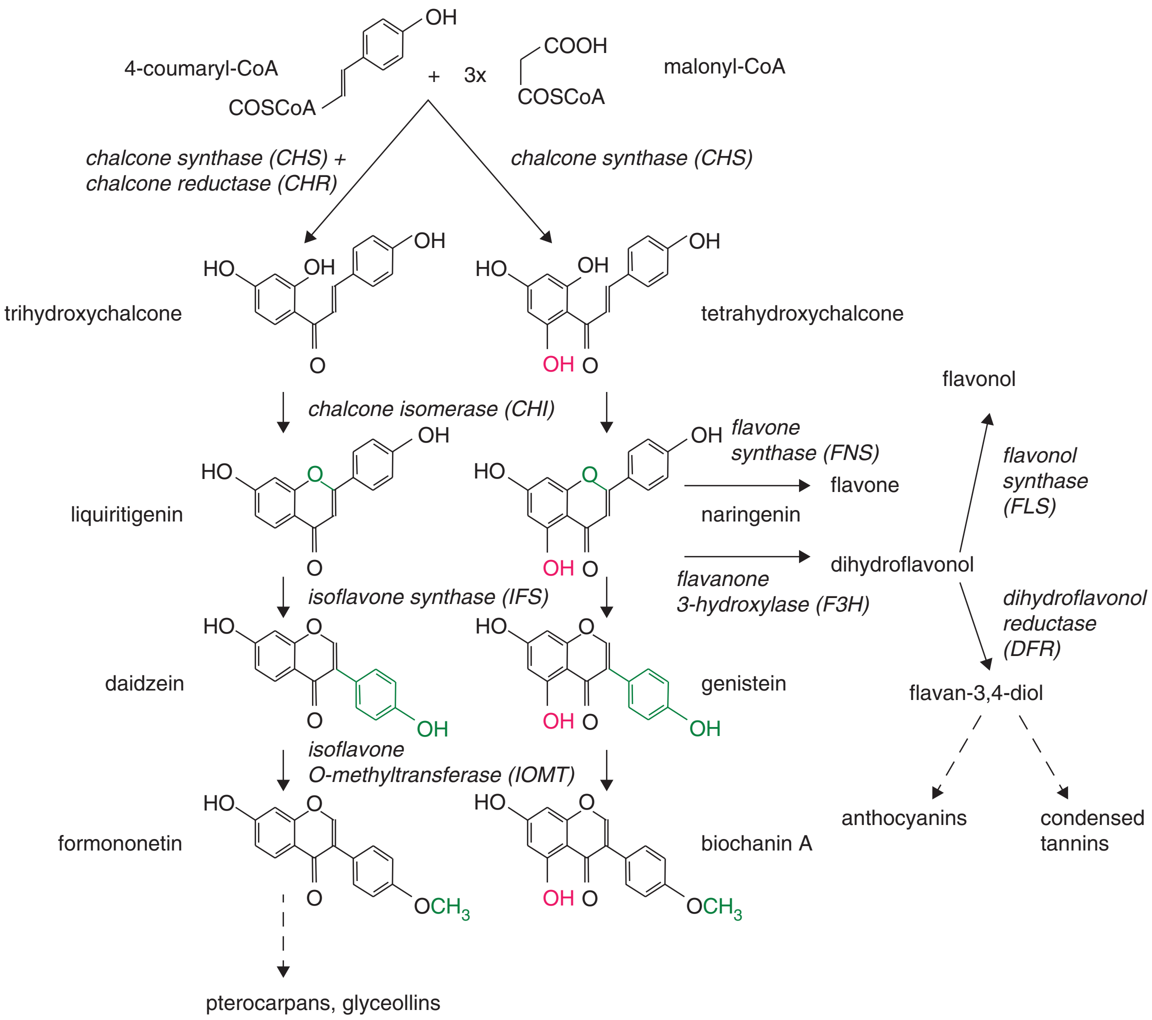


FIG. 1. Isoflavonoid biosynthetic pathway in legumes showing parallel processing of 5-reduced chalcones (left side) and 5-hydroxylated (right side) chalcones and isoflavones. Naringenin is the key branch point for producing isoflavonoids or other flavonoids such as flavonols, anthocyanins and condensed tannins. Enzymes and their abbreviations are labelled in italics; dashed arrows indicate several enzymatic reactions.

Noel, 2003). In addition, legumes also possess a chalcone reductase (CHR) which, together with CHS, can form a trihydroxychalcone (Bomati *et al.*, 2005). Tri- and tetrahydroxychalcones are converted by chalcone isomerases (CHIs) to the flavanones liquiritigenin and naringenin, respectively (Dixon *et al.*, 1988; Jez and Noel, 2002). Isoflavone synthase (IFS) subsequently converts liquiritigenin to daidzein, and naringenin to genistein (Hashim *et al.*, 1999; Sawada *et al.*, 2002). Methylation of daidzein and genistein by isoflavone-*O*-methyltransferases (IOMTs) leads to the formation of formononetin and biochanin A (He *et al.*, 1998; Liu and Dixon, 2001).

Genes coding for these enzymes have been cloned and functionally characterized from a range of legumes (for a review, see Rasmussen, 2009). However, no isoflavonoid pathway genes from white clover have been functionally assessed so far. As a start to exploring whether it is possible to produce high levels of isoflavonoids in white clover, a metabolic engineering approach was initiated, which focused on testing the functionality of an *IFS* gene cloned from white clover. As knowledge of the mechanisms of isoflavonoid biosynthesis enzyme function is limited, cloned isoflavonoid biosynthesis genes cannot be confirmed as being functional by sequence analysis alone (Deavours *et al.*, 2006). While *Nicotiana tabacum* (tobacco) is considered not to produce isoflavonoids (Yu *et al.*, 2000; Joung *et al.*, 2003; Liu *et al.*, 2007), it has been shown previously to accumulate isoflavonoids when expressing soybean *IFS* or a soybean/alfalfa *IFS/CHI* fusion gene (Tian and Dixon, 2006). We have therefore used this model system to test the functionality of a putative *IFS* gene cloned from white clover. It had also been shown that expression of soybean *IFS* in anthocyanin-accumulating tobacco tissues resulted in enhanced levels of isoflavonoid production (Yu *et al.*, 2000; Tian and Dixon, 2006). Anthocyanin biosynthesis can be induced in tobacco leaves by transformation with the R2R3 MYB transcription factor *ANT1* from tomato (Mathews *et al.*, 2003), and we tested here if the expression of white clover *IFS* in *ANT1*-expressing tobacco plants results in enhanced isoflavonoid production.

MATERIALS AND METHODS

Plant materials

Nicotiana tabacum ‘Wisconsin 38’ (‘W38’) seed was obtained from stocks held at Agresearch Grasslands (Palmerston North, New Zealand). Seeds were surface-sterilized in bleach (5 % available chlorine) for 20 min followed by five washes with sterile water. The seed was germinated and grown on MS0 nutrient agar without hornones (Murashige and Skoog, 1962) under sterile conditions in a growth room under 16 h d^{-1} of diffuse fluorescent lighting at 20 °C.

The white clover (*Trifolium repens*) cultivar 'Sustain' and the tomato (*Solanum lycopersicum*) variety 'Grosse Lisse' were grown outdoors and used for genomic DNA isolation.

Database query and identification

To obtain white clover *IFS* genes, 'isoflavone synthase' was used as a keyword to query the public NCBI (National Center for Biotechnology Information) nucleotide database (http://www.ncbi.nlm.nih.gov/). Fifty-one sequences from a range of species including white clover (GenBank accession nos AF195814 and AF195815; Jung *et al.*, 2000) were returned. One of the *IFS* sequences (AF195814) was used to query the Pastoral Genomics in-house GeneThresher® database, and two full-length *IFS* open reading frames of 1575 bp were assembled and translated into a 524 amino acid protein. The genomic sequence was arranged in two exons spanning a 122 bp intron. Primers (Supplementary Data Table S1) were designed to amplify full-length *IFS* sequences from genomic DNA isolated from the white clover cultivar 'Sustain'. Seven sequence variants were isolated, cloned and sequenced (data not shown). *IFS2_12* (GenBank accession no. JQ756454), which had an intron of 368 bp, was used for tobacco transformation.

Isolation of genomic DNA, PCR, cloning and sequencing

Genomic DNA was isolated from fresh or frozen plant tissues using a DNeasy Plant Mini kit (Qiagen, BioLab Ltd, Auckland, New Zealand) following the manufacturer's instructions. DNA preparations were treated with RNase A (Sigma NZ Ltd., Auckland, New Zealand) to remove RNA from the samples. The concentration and purity of DNA samples were assessed by determining the ratio of absorbance at 260 and 280 nm using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Thermo Scientific, DE, USA).

The reaction mixture (20 µL) for PCR amplification of *IFS2_12* contained 1× high fidelity PCR buffer, 2·5 U of Triple Master polymerase (Eppendorf, Hamburg, Germany), 0·2 mM of each dNTP, 0·4 µM of each primer (Supplementary Data Table S1) and 10 ng of DNA template. PCRs were performed in a thermocycler (MJResearch PTC200, ABI9700, ABI2720, or BioRad iCycler) using the following temperature profile: hot start at 94 °C for 2 min, 30 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 180 s, followed by 72 °C for 7 min and a 4 °C hold. The reaction mixture (50 µL) for PCR amplification of *ANT1* from tomato contained 1× Pfx50 PCR buffer, 5 U of Pfx50 Polymerase (Invitrogen), 2·5 mM $MgSO_4$, 0·3 mM of each dNTP, 0·4 µM of each primer and 10 ng of genomic DNA template. The temperature profile was as follows: 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 90 s, followed by 72 °C for 5 min and a 12 °C hold. PCR products were separated by agarose gel (1 % w/ v) electrophoresis and visualized by ethidium bromide staining. Bands were excised and DNA extracted from the gel slice using a MinElute Gel Extraction Kit (Qiagen) or by freezing in liquid nitrogen and subsequent centrifugation at 16 600 *g*. PCR products were cloned into the pENTR-D-TOPO vector (Invitrogen) and transformed into OneShot Top10 *Escherichia coli* cells by chemical transformation. Bacterial plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

Stable and transient transformation of tobacco

The Gateway-adapted binary vector pRSh1 was used to transform genes into tobacco. This contains, within the left and right borders of the transferred DNA, the BAR gene, which provides resistance to ammonium glufosinate herbicide (Thompson *et al.*, 1987), and a Gateway cloning site where the gene of interest is inserted. Both genes are driven by a *Cauliflower mosaic virus* (CaMV) 35S promoter. The white clover *IFS2_12* gene, the soybean–alfalfa *IFS/CHI* construct (Tian and Dixon, 2006) and the tomato *ANT1* gene (Mathews *et al.*, 2003) were cloned into pRSh1 (Supplementary Data Fig. S1) and used for stable and transient transformation of tobacco. The empty vector pRSh1 contains the *ccdB* gene which is lethal to *E. coli.* As this gene may adversely affect plant cells, it was not used as a vector control. The binary vector pHZbar was used as an empty vector control instead, containing only the *BAR* gene within the left and right borders. The binary vector pBin61 (Bendahmane *et al.*, 2000) contains the *P19* protein gene, from *Tomato bushy stunt virus*, which is a suppressor of post-transcriptional gene silencing (Voinnet *et al.*, 2003). Transformations were also performed using the pRS13 vector which contains green fluorescent protein (*GFP*) and β-glucuronidase (*GUS*) genes (Supplementary Data Fig. S2) as a visual control in the transient tobacco expression system.

To produce stable tobacco transformants, leaf discs from *N. tabacum* were transformed using *Agrobacterium*-mediated transformation and plant regeneration protocols as described in An (1985) and Horsch *et al.* (1985). The presence of the three transgenes was confirmed by PCR with appropriate primers (Supplementary Data Table S2, and Fig. S3).

Transient tobacco transformants were produced using the leaf infiltration method (Kapila *et al.*, 1997). An *A. tumefaciens* 'GV3101' strain carrying the desired vector was grown overnight in 20 mL of liquid MGL medium (Murashige and Skoog, 1962; without hormone supplement) containing 100 µg ml^{-1} spectinomycin or 50 µg ml^{-1} kanamycin, at 28 °C with shaking at 200 rpm. The bacteria in the broth were pelleted by centrifugation and the cells resuspended in 10 mL of sterile infiltration buffer (10 mM $MgCl_2$, 10 µM acetosyringone). The bacterial suspension was incubated for 2–3 h at room temperature and, for co-expression experiments separate bacterial suspensions were mixed together prior to infiltration. Tobacco leaves were infiltrated by pressing the suspension with a needleless syringe into the abaxial side of the leaf through a small incision. To determine the optimal concentration of bacterial suspensions, spatially separated leaf areas were infiltrated with six serial dilutions (1/1, 1/2, 1/4, 1/8, 1/16 and 1/32) and visually examined for anthocyanin production and leaf vitality (Fig. 2). The optimal tobacco tissue to infiltrate was the tissue between veins of the first fully expanded leaves developed approx. 3 weeks after transplanting small plants from peat plugs into pots in the greenhouse. Infiltrated areas were left for between 1 and 4 weeks (7 d unless mentioned otherwise) to give time for the transgene to be expressed and for metabolites to accumulate. Infiltrated areas were then dissected from the leaves with a scalpel, freeze-dried and weighed prior to isoflavonoid extraction.

Crossing of stable tobacco transformants

Mature tobacco plants transformed with white clover *IFS2_12* (Nt-IFS2_12), soybean/ alfalfa *IFS/CHI* (Nt-IFS/CHI) and *ANT1* (Nt-ANT1) that produced flowers at the same time were crossed in various combinations as well as self-fertilized. Crossed flowers were labelled with tags identifying the pollen donor, and mature seed pods were collected and stored at 4 °C. Seedlings were grown and leaf tissue collected from 3–6 young plants from each cross. The original Nt-ANT1 5P01963 plant and three ‘W38’ wild-type control plants were also sampled. Leaf tissue was freeze-dried, ground, and isoflavonoids extracted and analysed by liquid chromatography coupled to mass spectrometry (LCMS).

Isoflavonoid extraction and detection

Freeze dried (Flexi-Dry™ µP freeze drier) tobacco leaf samples were placed into 2 mL screw-top microtubes (Sarstedt) pre-loaded with five 3 mm steel balls or a 200 µL volume of 1 mm ceramic beads and ground in a Fast Prep FP120 mixer mill twice for 30 s at 4 m s^{-1}. Isoflavonoids were extracted from 150 mg of dry tissue three times with 1 ml of acetone for 30 min, extracts were centrifuged at 16 600 *g* for 1 min, supernatants combined and the acetone evaporated under nitrogen gas. Residues were dissolved in 200 µL of 80 % (v/v) methanol containing 0·1 % (v/v) acetic acid and samples mixed in an Eppendorf Comfort Thermomixer at room temperature with shaking at 1400 rpm for 30 min. Samples were centrifuged at 16 600 *g* for 10 min and the supernatants decanted into new tubes, stored at –20 °C and centrifuged again before transferring approx. 700 µL into glass vials.

Extracts were separated in a high-performance liquid chromatography (HPLC) system consisting of two JASCO X-LC 3085PU high pressure pumps (JASCO International Co. Ltd, Tokyo, Japan), and a HTS PAL autosampler with a 25 µL syringe and a 15k psi injection valve (CTC Analytics AG, Zwingen, Switzerland), coupled to a Thermo LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Thermo Finnigan Xcalibur software (version 2·1) was used for data acquisition and processing. A 20 µL injection of sample was made onto a Phenomenex Luna C18(2)-HST column (100 × 2·0 mm, 2·5 µm particle size; Phenomenex, Torrance, CA, USA). The HPLC solvents used were: solvent A = 0·1 % (v/v) formic acid in MilliQ® water; solvent B = 0·1 % (v/v) formic acid in acetonitrile (LiChroSolv grade, Merck, New Zealand). The flow rate was 400 µL min^{-1} and the solvent gradient used was: 0 min 5 % (v/v) B, 6 min 5 % (v/v) B, 11 min 10 % (v/v) B, 26 min 17 % (v/v) B, 31 min 23 % (v/v) B, 41 min 30 % (v/v) B, 45 min 50 % (v/v) B, 52 min 97 % (v/v) B, 59 min 97 % (v/v) B, 61 min 5 % (v/v) B, and 65 min 5 % (v/v) B. The acetonitrile concentration changed linearly between time points.

Mass spectra were acquired using electrospray ionization (ESI) in positive mode with a spray voltage of +4·5 kV, an ion source capillary temperature of 275 °C and the nitrogen sheath, auxiliary and sweep gas flow rates set to 20, 5 and 0 (arbitrary units), respectively. The first 3 min of flow from the HPLC were diverted to waste. The 61 min run protocol was used and the LCMS was set to scan for MS^1 ions of *m/z* 271 (for genistein), 433 (for genistein glycoside = genistin) and 519 (for genistin malonate), MS^2 fragments of *m/z* 271 and MS^3 fragments of *m/z* 153. LCMS peaks were identified and quantified by comparison with a genistein standard (Sigma) for elution time, mass spectrum and Retro-Diels-Alder (RDA) fragment masses of collision energy fragmentations performed in the trap with helium.

Quantification was carried out by creating a calibration curve of the chemical standard of known concentration, using MS ion count peak areas. Quantification of the peaks was based on ion counts of fragmented *m/z* 271 ions, and the concentrations and weights of genistein and its conjugates were measured in ‘genistein aglycone only’ units. In other words, the weights do not include the added weights of the glucoside or glucoside-malonate molecules. The true weight of genistin, including the glucose, in the sample can be calculated by multiplying the measured weight by 433/271 = 1·60 times. Likewise the genistin malonate results can be multiplied by 519/271 = 1·92 times to obtain the true weight. The MS^2 ion count peak area was compared with that of known aglycone quantity standards to quantify the isoflavonoid content in the sample.

RESULTS

Optimization of tobacco leaf infiltration

Mature young wild-type tobacco leaves were infiltrated with *A. tumefaciens* transformed with the R2R3 MYB transcription factor *ANT1* from tomato (Mathews *et al.*, 2003) which induces anthocyanin biosynthesis resulting in red tissue coloration. To optimize the infiltration method, bacterial suspensions were serially diluted and infiltrated into spatially separated leaf areas (Fig. 2). Anthocyanins were clearly visible 7 d post-infiltration as a speckled red tinge in the *ANT1* infiltrations of wild-type tobacco plants, with 1/8 and 1/16 dilutions giving the darkest colour change compared with the other dilutions (Fig. 2A, E). As an additional control, wild-type tobacco leaves were also infiltrated with *A. tumefaciens* transformed with *GFP* and *GUS* genes. Leaves infiltrated with *GFP* were microscopically analysed for the presence of GFP which accumulated in cells within the infiltrated areas but not in non-infiltrated areas (Supplementary Data Fig. S4).

The binary vector pBin61 contains the *P19* protein gene, from *Tomato bushy stunt virus*, which is a suppressor of post-transcriptional gene silencing (Bendahmane *et al.*, 2000). Voinnet *et al.* (2003) had shown previously that co-infiltration of *P19* with *GFP* into *N. benthamiana* leaves resulted in an increase in *GFP* expression compared with infiltration of *GFP* alone. We therefore also tested the effects of combinations of *ANT1* + *P19* and *GFP* + *P19*. Comparison of the four different infiltrates showed the effect of P19 on tobacco leaves (Fig. 2). The *ANT1*- and *GFP*-infiltrated areas without *P19* remained

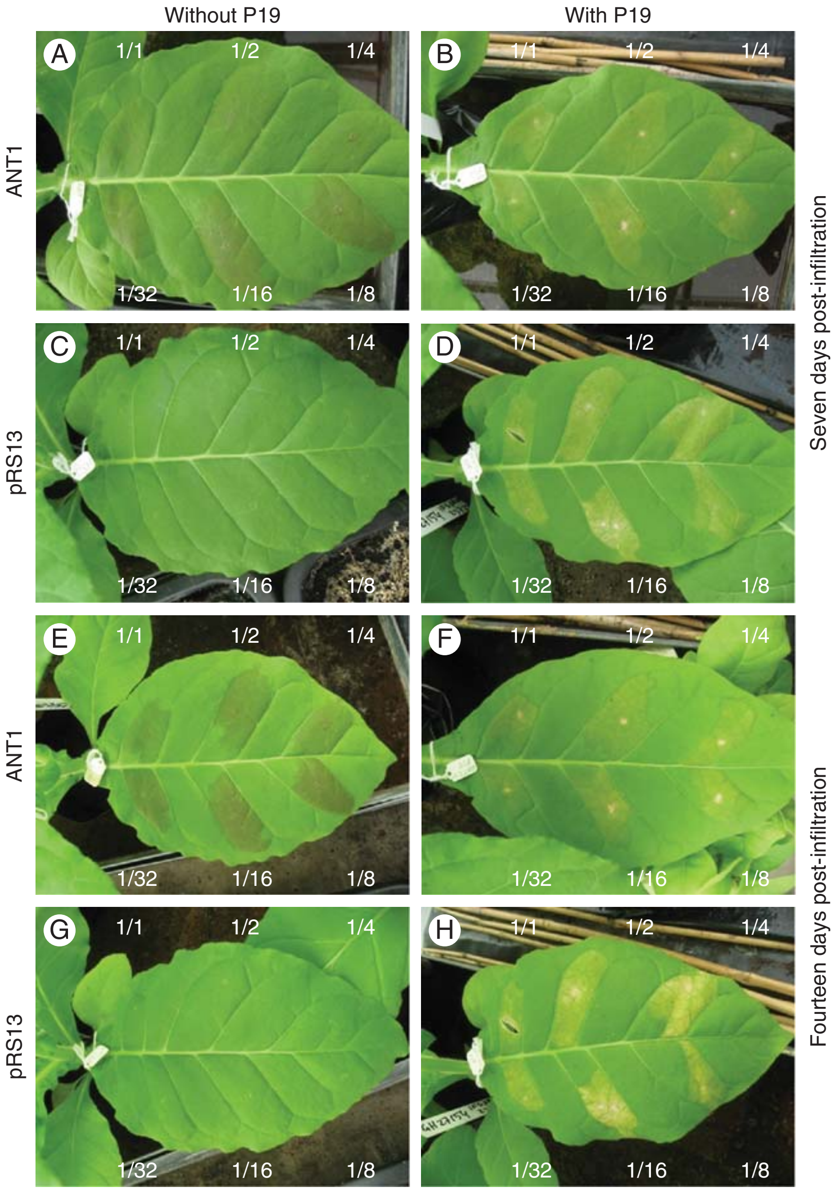


FIG. 2. Serial dilution of *A. tumefaciens* tobacco leaf infiltrates of *ANT1* or pRS13 (contains GFP) without (left hand panels) or with (right hand panels) P19, at 7 d (upper panels) and 14 d (lower panels) post-infiltration in wild-type tobacco plants. The dilutions of the infiltrate are labelled around each leaf.

healthy throughout the life of the leaf (Fig. 2, left hand panels), while *ANT1* + *P19*- and *GFP* + *P19*-infiltrated areas both turned yellow at 7 d post-infiltration at all dilutions (Fig. 2, right hand panels). The *GFP* + *P19* infiltrations even became necrotic in some areas, and at 14 d post-infiltration this effect was more pronounced (Fig. 2H). Clearly the presence of *A. tumefaciens* carrying the P19 plasmid was detrimental to the infiltrated leaf areas.

Stable transformation of tobacco with ANT1

The isoflavonoid and anthocyanin pathways share common precursors such as naringenin. To increase precursor supply, we stably transformed tobacco plants with the R2R3 MYB transcription factor *ANT1* from tomato (Mathews *et al.*, 2003), thus increasing flux through the phenylpropanoid pathway. Ten tobacco plants transformed with *ANT1* were regenerated and named Nt-ANT1 in the following text, and the presence of the transgene was confirmed using PCR (Supplementary Data Fig. S3). The Nt-ANT1 plants had a range of phenotypes from mainly green to uniformly bronze or darker purple coloured (Fig. 3). Many plants had intermediate colour levels with red vasculature (Fig. 4A). Root phenotypes of young regenerants growing on agar plates developed from colourless young roots to dark red root tips and eventually turning over to completely red roots (Fig. 4B). Some regenerants also had leaves with both green and purple areas simultaneously, even though PCR analysis demonstrated the presence of the transgene in both types (Supplementary Data Fig. S3C). Nt-ANT1 plants had a growth habit, form and vigour similar to those of wild-type plants, but the most intensely coloured plants were smaller compared with the wild type. Flowers of Nt-ANT1 plants were more intensely pink and purple coloured than the light pink wild type, and seeds were darkly coloured compared with the light brown colour of wild-type seed (data not shown).

Isoflavonoid production in infiltration experiments

Stable Nt-ANT1 tobacco transformants were used for infiltration experiments to determine the functionality of the white clover *IFS2_12* gene. We also used a soybean/alfalfa *IFS/CHI* construct, which had been shown previously to result in isoflavonoid production in tobacco (Tian and Dixon, 2006; Nt-IFS/CHI), as a positive control with or without *ANT1* infiltration. In a preliminary study, we infiltrated Nt-ANT1 plants with the *IFS/CHI* construct and co-infiltrated the precursor naringin (naringenin rhamnoglucoside). Interestingly, Nt-ANT1 leaf areas infiltrated with *IFS/CHI* and naringin showed noticeably reduced pigment levels and were greener than the bronze red colour of the surrounding leaf (Fig. 5), indicating reduced production of anthocyanins probably due to competition for substrates between flavanone 3-hydroxylase (F3H) and the IFS/CHI enzymes. However, no peaks corresponding to isoflavonoids were detected by HPLC-photodiode array (PDA) which might have been due to the relatively low sensitivity of PDA detection. We therefore developed an LCMS^n-based method allowing for detection of much lower concentrations of isoflavonoids.

Tobacco plants transformed with the soybean/alfalfa *IFS/CHI* construct had been shown previously to accumulate the isoflavonoids genistein, its glycosylated conjugate genistin, as well as genistin malonate (Tian and Dixon, 2006). To detect these compounds, the LCMS was set to scan for MS^1 ions and for MS^2 fragments specific for genistein, genistein glucoside and genistin malonate. When leaves of Nt-ANT1 plants were infiltrated with *IFS2_12* or *IFS/CHI*, or Nt-IFS/CHI plants were infiltrated with or without *ANT1*, all of the samples which had an *IFS* gene present (as either a stable or a transient transgene) produced MS^2 peaks of *m/z* 271 and MS^3 peaks of *m/z* 153 (Fig. 6), confirming that genistein and its conjugates were produced in these tissues. No genistein or genistein conjugates were detected in tobacco plants that were not transformed with an *IFS* gene, confirming that tobacco does not produce isoflavonoids, as has been shown in other studies (Tian and Dixon, 2006; Liu *et al.*, 2007).

Genistin was the major genistein component, being present at 2–9 times the concentration of genistin malonate. The genistein aglycone was present in concentrations too low to be quantifiable (data not shown). No genistein or genistein conjugates were detected in a no-sample extraction or in any samples that did not contain an *IFS* gene, i.e. the NT-ANT1 plants without infiltration or infiltrated with the pHZbar empty vector control. The levels of genistin and genistin malonate in Nt-ANT1 leaf tissues infiltrated with white clover *IFS2_12* or the soybean/alfalfa *IFS/CHI* construct ranged from 0·07 to 1·26 μg g^{-1} d. wt (Fig. 7). The two stably transformed Nt-IFS/CHI plants produced genistein without any infiltrate at lower levels of 0·04–0·15 μg g^{-1} d. wt. When infiltrated with *ANT1*, the amount of genistein conjugates increased, reaching very high levels in plant 5P02034 of 4·52 μg g^{-1} d wt. Concentrations of genistein conjugates were below the detection level in Nt-IFS/CHI leaves infiltrated with the pHZbar empty vector control.

Isoflavonoid content in progeny of stable tobacco transformant crosses

Once mature, tobacco plants transformed with white clover *IFS2_12* (Nt-IFS2_12), soybean/alfalfa *IFS/CHI* (Nt-IFS/CHI) and *ANT1* (Nt-ANT1) were either self-fertilized or crossed in various combinations. Seedlings were grown from each cross. Isoflavonoids were extracted from leaves of young plants and analysed by LCMS. MS^n fragmentation analysis confirmed the presence of genistein in the forms of genistein, genistin and genistin malonate in a number of transgenic plants (Fig. 8).

Quantification of the three compounds again found genistin to be the most common form (95–100 % of the total genistein

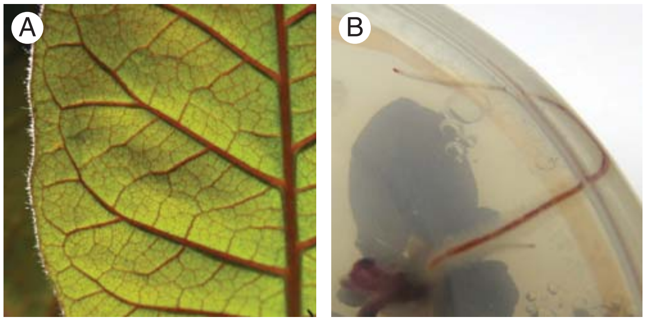


FIG. 4. Nt-ANT1 regenerants showing (A) red vasculature and (B) roots.

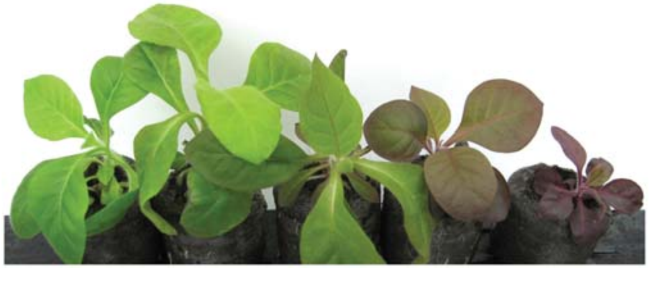


FIG. 3. Nt-ANT1 regenerants showing the diversity of colour phenotypes.

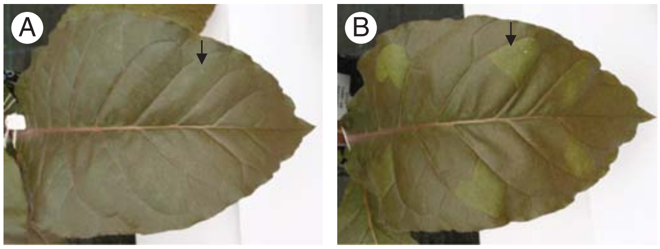


FIG. 5. Nt-ANT1 leaves infiltrated with infiltration solution only (A) or *A. tumefaciens* carrying an *IFS/CHI* fusion construct (B) showing the loss of colour in the *IFS/CHI* infiltration area.

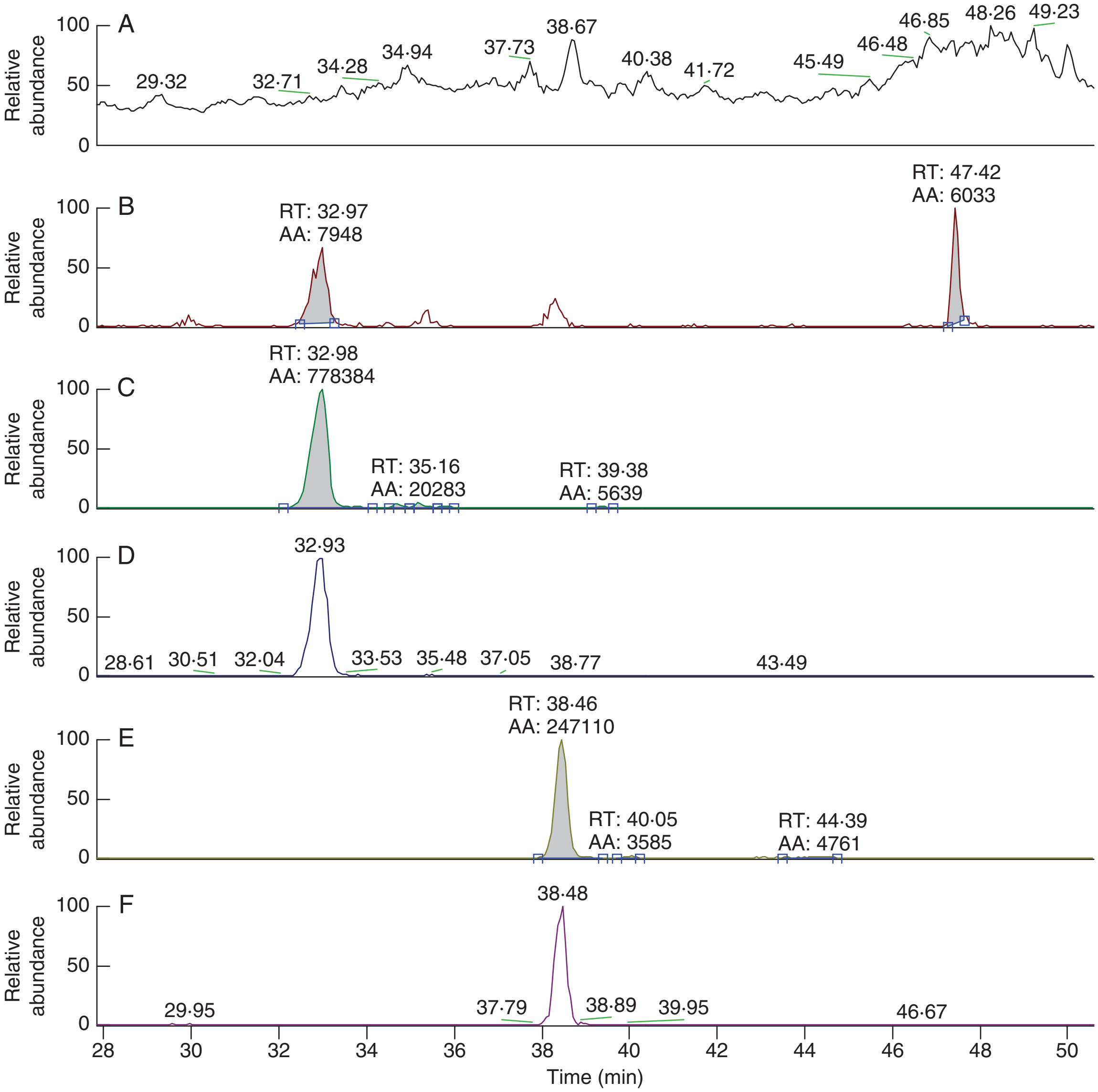


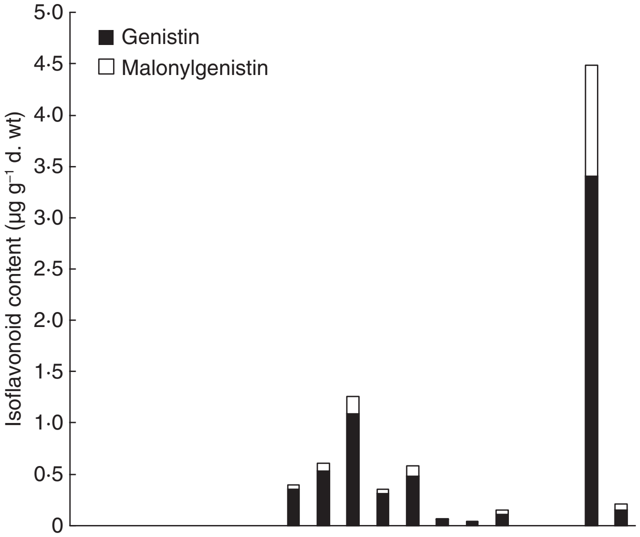
FIG. 6. LCMS spectra of leaf tissue extracts from the Nt-IFS/CHI transformant 5P02034 infiltrated with *ANT1*. (A) MS^1 total ion count of all masses over retention times of 28–50 min. (B) Genistein and genistein conjugates – MS^2 fragments of *m/z* 153 from MS^1 ions of *m/z* 271. (C) Genistin – MS^2 fragments of *m/z* 271 from MS^1 ions of *m/z* 433. (D) Genistin – MS^3 fragments of *m/z* 153 derived from the MS^2 fragments in (C). (E) Genistin malonate – MS^2 fragments of *m/z* 271 from MS^1 ions of *m/z* 519. (F) Genistin malonate – MS^3 fragments of *m/z* 153 derived from the MS^2 fragments in (E).

conjugate content), while genistein was the second most prevalent at concentrations of 1/20th to 1/200th of the amount of genistin. Unlike in the infiltration experiment, genistin malonate was present in very low concentrations, at approximately only a quarter of that observed for genistein (data not shown).

All of the plants that contained no transgene or only *ANT1* produced no detectable genistein or genistein conjugates. Of the 30 plants that were progeny of IFS2_12 or IFS/CHI transformants, 13 produced a detectable level of genistin ranging from 0·006 to 19·2 μg g^{-1} d. wt (Fig. 9). The highest level was detected in a purple plant arising from a cross between Nt-ANT1 and Nt-IFS/CHI, while the second highest concentration was found in a plant derived from a selfed Nt-IFS2_12 tobacco plant (Fig. 9). There was no clear correlation between genistin levels and the colour phenotype.

DISCUSSION

Selection of a suitable model plant to assess functionality of cloned genes requires a plant amenable to transformation, and one that does not normally produce the key metabolites. To characterize functionally a white clover gene putatively coding for an IFS isolated in this study, we expressed *IFS2_12* in tobacco, and analysed transformed plants for isoflavonoid production by LCMS^n. Tobacco was chosen as it is readily transformed and generally accepted as a model species which does not produce isoflavonoids naturally (Joung *et al.*, 2003; Liu *et al.*, 2007). It had previously been shown to produce the isoflavonoid genistein and its conjugates following transformation with an *IFS/CHI* gene fusion construct, while untransformed plants did not accumulate any detectable levels



Plant	Stable transgene	Infiltrated transgene
No-sample extraction		
5P01965	ANT1	None
5P01969	ANT1	None
5P01967	ANT1	None
5P01965	ANT1	Control
5P01969	ANT1	Control
5P01967	ANT1	Control
5P01965	ANT1	IFS2_12
5P01969	ANT1	IFS2_12
5P01967	ANT1	IFS2_12
5P01965	ANT1	IFS-CHI
5P01969	ANT1	IFS-CHI
5P01967	ANT1	IFS-CHI
5P02034	IFS-CHI	None
5P02035	IFS-CHI	None
5P02034	IFS-CHI	Control
5P02035	IFS-CHI	Control
5P02034	IFS-CHI	ANT1
5P02035	IFS-CHI	ANT1

FIG. 7. Genistin and genistin malonate content of tobacco plants with combinations of stable and infiltrated *IFS*, *ANT1* and negative control (pHZbar) transgenes. The aglycone genistein is not shown because its concentrations were too low.

of the isoflavonoids (Yu *et al.*, 2000; Tian and Dixon, 2006). A major finding of our study was that both transient and stable expression of white clover *IFS2_12* results in the production of genistein conjugates in tobacco leaves (Figs 6–8), confirming the functionality of the *IFS2_12* gene and showing that its expression is necessary and sufficient for producing genistein in a naïve model plant. Analysis of progeny from crosses of Nt-ANT1 and Nt-IFS/CHI or selfed Nt-IFS2_12 plants also showed that the transgenes were inherited and isoflavonoids accumulated in progeny (Fig. 9).

Here, we also used leaf infiltration to express transiently clover *IFS2_12*, *IFS/CHI* fusion (Tian and Dixon, 2006) and the R2R3 MYB transcription factor *ANT1* from tomato (Mathews *et al.*, 2003). This method is very rapid, with anthocyanin and isoflavonoid synthesis being observable after only 7 d (Figs 3 and 4). Quantities of genistein conjugates accumulating in tobacco plants transiently expressing IFS genes were similar to those in stably transformed plants, making this a viable functional testing technique that is faster than producing stable transformants (Figs 5 and 6). The P19 protein had previously been shown to suppress post-transcriptional gene silencing and thus increase the production of transiently expressed transgene products and also maintain this production until senescence of the leaf in *N. benthamiana* (Voinnet *et al.* 2003). However, *N. tabacum* leaves used in this study infiltrated with P19 turned yellow, while those without P19 remained green and healthy, showing that the P19 protein itself or some other factor of the P19 vector was toxic (Fig. 2).

Tobacco plants expressing *ANT1* showed variable coloured phenotypes ranging from mainly green to dark purple (Fig. 3). It had been shown previously that *ANT1* transcript levels correlated well with the concentrations of anthocyanins in both tomato and tobacco overexpressing *ANT1* (Mathews *et al.*, 2003), and it is possible that the phenotypic variation seen here is due to variable transcript levels. In our study, *ANT1* expression was under the control of the cauliflower mosaic virus 35S promoter which is expressed in all tissues. Anthocyanin accumulation patterns derive from *ANT1* expression levels as well as the presence of repressors and interactions of the *ANT1* MYB with compatible, endogenous, differentially expressed, basic helix–loop–helix (bHLH) factors and WD-repeat proteins to form a transcriptional activation complex (Albert *et al.*, 2011). Our findings of dark red vasculature tissues and root tips may reflect this expression of endogenous factors (Fig. 4). It had been suggested previously that plant growth and flavonoid production might be inversely correlated (Hofmann *et al.*, 2003), and we also saw reduced growth of plants accumulating very high levels of anthocyanins (Fig. 3). Some flavonoids have been shown to regulate auxin transport, a phytohormone involved in plant growth regulation (Taylor and Grotewold, 2005), and a direct link between high levels of flavonoids and reduced growth due to inhibition of auxin transport has been shown in *A. thaliana* plants suppressed in lignin biosynthesis (Besseau *et al.*, 2007).

The tobacco plants were either infiltrated or transformed with the R2R3 transcription factor *ANT1* to create plants that were likely to accumulate higher levels of a precursor common to the biosynthetic pathways leading to isoflavonoids and anthocyanins, namely naringenin. Naringenin had been shown previously by Liu *et al.* (2002) to be a limiting factor in genistein production in *Arabidopsis thaliana* plants transformed with a soybean *IFS*. When *IFS* was introduced into an *A. thaliana tt6/ tt3* (transparent testa) double mutant, which has structural defects in both flavanone 3-hydroxylase (F3H) and dihydroflavonol reductase (DFR), and produces much lower concentrations of flavonoids and anthocyanins, genistein levels were much higher than those produced in the non-mutant transformed plants (Liu *et al.*, 2002). This demonstrated that a limiting factor in genistein production was competition for naringenin between IFS and F3H. Consistent with those results, we found here that tobacco plants expressing *IFS/CHI* and *ANT1* simultaneously accumulated higher levels of genistein conjugates compared with plants transformed with the fusion construct only (Fig. 7).

The fact that the compounds detected in the tobacco plants transformed with *IFS* were almost entirely genistin and genistin malonate shows that endogenous glucosyl and malonyl transferases are active in tobacco and can add glucose and malonate groups to the aglycone genistein. Glycosylated isoflavonoids are more water soluble than the aglycone, and glycosylation is required for their transport into the vacuole (Jones and Vogt, 2001). We did not see production of any other isoflavonoids such as daidzein, formononetin or

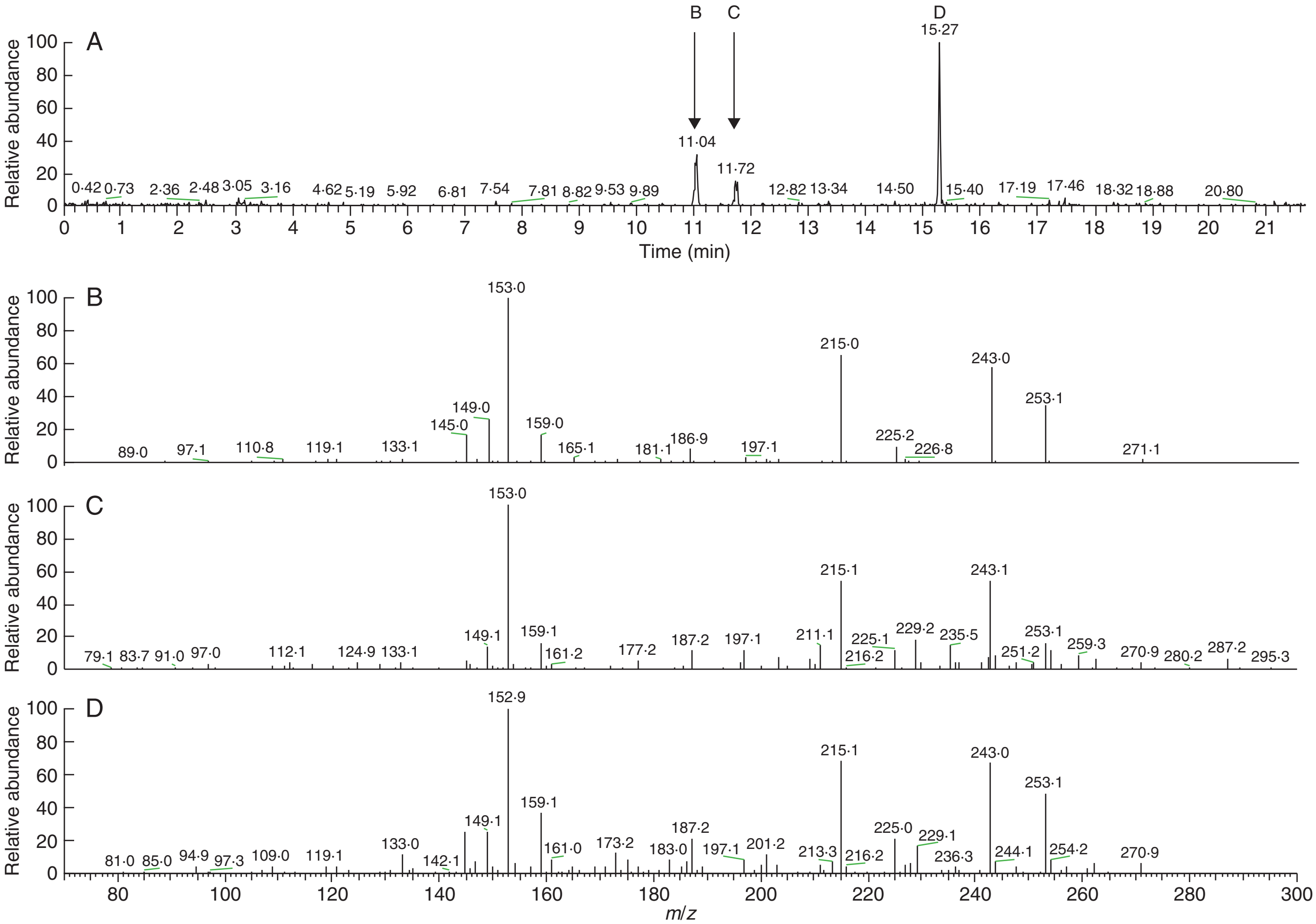
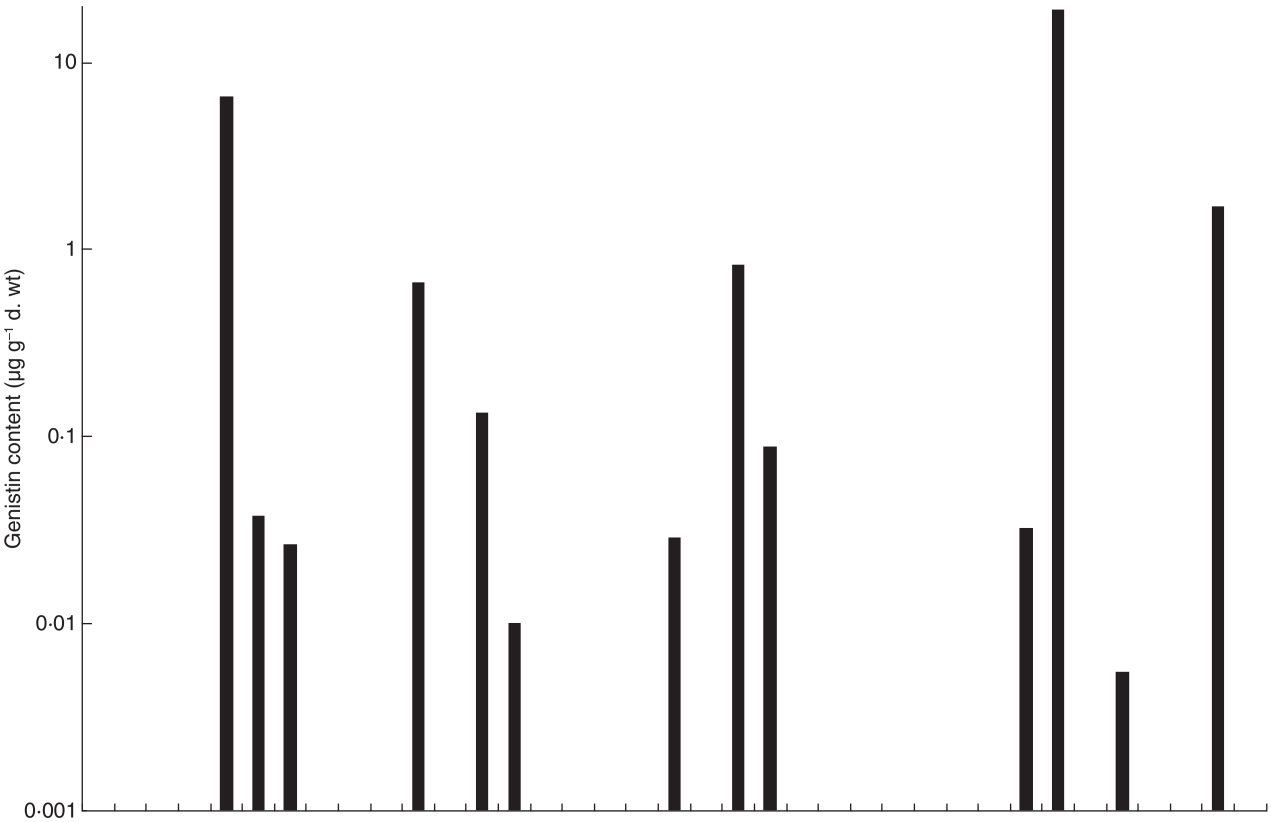


FIG. 8. Mass spectra of three peaks containing MS^2 fragments of *m/z* 271 in a leaf tissue extract from a selfed offspring of tobacco plant 23722 expressing the white clover *IFS2_12* transgene. (A) MS^3 fragments of *m/z* 153 derived from MS^2 fragments of *m/z* 271 over the retention times of 0–21 min. (B) Genistin: peak in (A) and MS^3 mass spectrum of that peak. (C) Genistin malonate: peak in (A) and MS^3 mass spectrum of that peak. (D) Genistein: – peak in (A) and MS^3 mass spectrum of that peak.

biochanin A, indicating that tobacco lacks genes coding for *CHR* and *IOMT*.

When bronze-coloured *ANT1* leaves were infiltrated with *IFS/CHI*, the infiltrated areas were less red-coloured after 7 d compared with the surrounding uninfiltrated areas or the areas infiltrated with buffer only (Fig. 5). This indicated that a constituent of the infiltrate was reducing the anthocyanin concentration. It is possible that the presence of *A. tumefaciens* had a general effect on the tissues with a side effect of reducing anthocyanin content. Alternatively, the introduction of *IFS/CHI* into these cells may have resulted in a depletion of naringenin, the common precursor to isoflavonoids and anthocyanins, and thus caused a reduced production and accumulation of anthocyanins which may, as a result of turnover, actually decrease in concentration. Xie *et al.* (2006) produced transgenic tobacco plants expressing simultaneously the MYB transcription factor *PRODUCTION OF ANTHOCYANIN PIGMENT 1* (*PAP1*) and anthocyanidin reductase (*ANR*) to produce condensed tannins. ANR reduces anthocyanins to *cis*-flavan-3-ols, monomeric precursors of condensed tannins (proanthocyanidins), and competes with anthocyanidin glycosyltransferases which produce glycosylated anthocyanins. Plants expressing *PAP1* and *ANR* transgenes contained lower levels of anthocyanins than those expressing only PAP1 in that study.

White clover plants accumulating high levels of isoflavonoids would be useful in pastoral animal production systems as these compounds can protect clover plants from insect attack. Conventional breeding of white clover with high levels of isoflavonoids has not been successful in the past, and metabolic engineering with known pathway genes might be an alternative strategy. The functional characterization of a white clover *IFS* gene confirms that its constitutive expression results in increased accumulation of isoflavonoids in a heterologous system, and these findings pave the way for the production of white clover cultivars with modified isoflavonoid levels. However, formononetin is demethylated and metabolized to equol by micro-organisms in the rumen which acts as a phyto-oestrogen in grazing mammals and can reduce fertility in sheep if consumed in large quantities (Beck, 1964). It might therefore be necessary to develop a strategy for white clover which simultaneously upregulates *IFS* while downregulating *CHR* to increase flux into the biosynthesis of genistein and biochanin A, which, in contrast to formononetin, are only mildly oestrogenic. Our study shows that the tobacco model system is suitable for functional characterization of white clover biosynthetic



Seed from	Maternal transgene	Paternal transgene	Plant colour
5P01963 (original plant)	ANT1		Purple
5P03265	None		Green
5P03265	None		Green
5P03265	None		Green
23722	IFS2_12	Selfed	Green
23722	IFS2_12	Selfed	Green
23722	IFS2_12	Selfed	Green
5P01963	ANT1	Selfed	Purple
5P01963	ANT1	Selfed	Bronze
5P01963	ANT1	Selfed	Bronze
5P02034	IFS-CHI	Selfed	Green
5P02034	IFS-CHI	Selfed	Green
5P02034	IFS-CHI	Selfed	Green
5P02035	IFS-CHI	Selfed	Green
5P02035	IFS-CHI	Selfed	Green
5P02035	IFS-CHI	Selfed	Green
5P02036	IFS-CHI	Selfed	Green
5P02036	IFS-CHI	Selfed	Green
5P02036	IFS-CHI	Selfed	Green
5P02034 × 5P01963	IFS-CHI	ANT1	Green
5P02034 × 5P01963	IFS-CHI	ANT1	Green
5P02034 × 5P01963	IFS-CHI	ANT1	Green
5P02036 × 5P01963	IFS-CHI	ANT1	Purple
5P02036 × 5P01963	IFS-CHI	ANT1	Purple
5P02036 × 5P01963	IFS-CHI	ANT1	Green
5P02036 × 5P01963	IFS-CHI	ANT1	Green
5P02036 × 5P01963	IFS-CHI	ANT1	Purple
5P02036 × 5P01963	IFS-CHI	ANT1	Purple
5P01963 × 5P02034	ANT1	IFS-CHI	Green
5P01963 × 5P02034	ANT1	IFS-CHI	Green
5P01963 × 5P02034	ANT1	IFS-CHI	Purple
5P01963 × 5P02034	ANT1	IFS-CHI	Green
5P01963 × 5P02035	ANT1	IFS-CHI	Green
5P01963 × 5P02035	ANT1	IFS-CHI	Green
5P01963 × 5P02035	ANT1	IFS-CHI	Purple
5P01963 × 5P02035	ANT1	IFS-CHI	Purple
5P01963 × 5P02035	ANT1	IFS-CHI	Purple

FIG. 9. Genistin content of seedlings from crossings of Nt-ANT1, Nt-IFS2_12 and Nt-IFS/CHI plants. A logarithmic scale was used to visualize the four orders of magnitude of genistin content. For the sake of clarity, the minor components, genistein and genistin malonate, are not shown.

genes and paves the way for analysis of additional isoflavonoid genes such as *CHR* and *IOMT* from white clover.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: primers used to isolate *IFS* from genomic DNA of *Trifolium repens*. Table S2: primers used to test for transgenes in *Nicotiana tabacum*. Figure S1: vector diagram of pRSh1. Figure S2: vector diagram of pRS13. Figure S3: gel photographs of PCR to confirm the presence of transgenes in *N. tabacum*. Figure S4: GFP expression in wild-type tobacco leaves infiltrated with pRS13.

ACKNOWLEDGEMENTS

This work was supported by a FRST NZ (Foundation for Research, Science and Technology) Enterprise Scholarship UOCX04009 to B.K.F. and by the Pastoral Genomics Research Consortium (VLAC0201). Pastoral Genomics is funded by DairyNZ, Beef + Lamb New Zealand, Fonterra, DEEResearch, AgResearch and the Ministry of Science and Innovation. We thank Dr R.A. Dixon (Noble Foundation, OK, USA) for providing us with the IFS/CHI fusion construct.

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